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Synthesis of the small subunit of ribulose-bisphosphate carboxylase from genes cloned into plasmids containing the SP6 promoter

Shirley ANDERSON and Steven M. SMITH*
Department of Botany, University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, U.K.

DNA sequences encoding ribulose 1,5-bisphosphate carboxylase small subunit precursor from *Pisum sativum* L. have been transcribed from plasmids containing the SP6 promoter, and translated in a wheat germ cell-free system. The small subunit precursor polypeptide, its *N*-terminal leader sequence (transit peptide) and the mature small subunit have each been synthesized independently from three different plasmid constructs. The precursor polypeptide is imported into isolated pea chloroplasts and processed to the mature small subunit by a stromal proteinase. The mature polypeptide is neither imported, nor subject to proteolysis by stromal extracts. The transit peptide alone is very rapidly degraded by a stromal proteinase activity which can be inhibited by EDTA or 1,10-phenanthroline. The use of these gene constructs helps to establish the crucial role of the transit peptide in protein import into the chloroplast.

INTRODUCTION

The synthesis of the chloroplast enzyme ribulose-1,5-bisphosphate (RuBP) carboxylase (EC 4.1.1.39) is of particular interest because it provides a unique opportunity to study the direct co-operation of nuclear and cytoplasmic genetic systems in synthesizing a protein comprised of two subunit types (Ellis, 1985). In this respect it provides a relatively simple model for understanding such nuclear–cytoplasmic interactions. The enzyme is comprised of eight large subunit polypeptides (L; M_r 53000) encoded in chloroplast DNA and eight small subunit polypeptides (S; M_r 14000) encoded by nuclear genes (Ellis, 1981). The S polypeptide is synthesized in the cytosol as a precursor (pS) of M_r 20000, from which 57 *N*-terminal amino acid residues (in the case of *Pisum sativum*) are removed during, or soon after, import into the chloroplast (Cashmore *et al.*, 1985). The mechanism of assembly of the S and L polypeptides is not known, but might require the participation of a third polypeptide (Ellis, 1985; Milos & Roy, 1985).

The *N*-terminal amino acid sequence of pS was termed the transit peptide (TP) because of its assumed function in directing S into the chloroplast (Chua & Schmidt, 1979). Subsequently it has been demonstrated that TP is capable of directing the import of a foreign protein, neomycin phosphotransferase II, into chloroplasts (Van den Broeck *et al.*, 1985). We are interested to learn more about the function of TP in the import process, and about its fate after import has taken place. We have chosen to synthesize pS, S and TP in cell-free systems employing SP6 RNA polymerase (Melton *et al.*, 1984) and wheat germ extract (Roberts & Paterson, 1973) in order to investigate the contribution which S and TP each makes to the function of pS. We have cloned the appropriate DNA sequences into plasmid vectors containing the SP6 promoter (Melton *et al.*, 1984) and established simplified procedures for their transcription and translation *in vitro*. We find that removal of TP from pS abolishes the ability of chloroplasts to import S, and that TP is subject to rapid degradation by a stromal proteinase activity.

MATERIALS AND METHODS

Reagents and enzymes

L-[^{35}S]Methionine (1200 Ci/mmol) and [^{32}P]dCTP (410 Ci/mmol) were obtained from Amersham International. Amino acids, trypsin and 1,10-phenanthroline were from Sigma Chemical Co., Percoll and m^{7}GpppG were from Pharmacia and other nucleotides from Boehringer Corp. SP6 RNA polymerase was from Anglian Biotechnology, P&S Biochemicals or New England Biolabs. Other enzymes were from Northumbrian Biotechnology, Amersham International or Boehringer Corp.

Cloned DNA sequences encoding S

The cDNA clone used as starting material for the synthesis of S is pGR407 (Figs. 1*a* and 1*b*). This clone was constructed from two incomplete cDNA clones (pSSU1 and pSSU69; Bedbrook *et al.*, 1980) since we did not have a single cDNA sequence encoding the entire S polypeptide. The construct was made by joining the 5′ end of pSSU69 to the 3′ end of pSSU1 at the *Sau*3A site located at codon 24 of the S polypeptide, and a *Bam*H1 linker was ligated to the 3′ end. The sequence is contained in pBR322 between *Hin*dIII and *Bam*H1 sites. The sequence of the pGR407 cDNA was determined by means of the dideoxynucleotide chain termination method (Sanger *et al.*, 1980). Specific DNA fragments were cloned into M13 vectors mp18 and mp19 (Yanisch-Perron *et al.*, 1985) using restriction endo-

Abbreviations used: bp, base pairs; RuBP, ribulose 1,5-bisphosphate; L, large subunit of RuBP carboxylase; pS, precursor to the small subunit of RuBP carboxylase; S, small subunit of RuBP carboxylase; TP, transit peptide; m^{7}GpppG, 7-methylguanosine(5′)triphospho(5′)guanosine.

* To whom correspondence and reprint requests should be addressed.

nucleases known to cleave the cDNA (three *Hin*dIII sites, three *Taq*1 sites and one *Bam*H1 site). In this way sequences were determined completely for both DNA strands. The coding information for S was sub-cloned as an *Sph*1 fragment into pUC18 (Yanisch-Perron *et al.*, 1985) and from there as a *Pst*1–*Bam*H1 fragment into pSP64 (Melton *et al.*, 1984), producing pSMS58 (Fig. 1*b*).

Cloned DNA sequences encoding pS and TP

We obtained the coding information for TP from a genomic clone of *Pisum sativum*, pgSSU47H (T. Gallagher, P. Gilmartin & R. J. Ellis, unpublished work). This *Hin*dIII–*Sph*1 fragment contains 206 bp, both strands of which were sequenced (Sanger *et al.*, 1980) after transfer to M13 vectors mp18 and mp19 (Yanisch-Perron *et al.*, 1985). To construct a plasmid directing the synthesis of pS, the *Hin*dIII–*Sph*1 fragment encoding TP, and the *Sph*1–*Bam*H1 fragment encoding S (from pGR407) were purified and ligated into pSP64 which had been digested with *Hin*dIII and *Bam*H1. The resulting plasmid is designated pSMS64 (Fig. 2*b*). Subsequently we obtained (from Dr. P. Turner, Biochemistry Department, Liverpool University) a derivative of pSP64 from which an unwanted *Sph*1 site had been deleted, and the cloning site polylinker replaced by that from pUC19. Into this plasmid (pSP6419) we inserted the *Hin*dIII–*Sph*1 fragment encoding TP, to produce pSPTP19 (Fig. 2*b*).

Transcription and translation

Supercoiled plasmids were prepared from cleared lysates of *Escherichia coli* by two cycles of centrifugation to equilibrium in CsCl/ethidium bromide gradients (Maniatis *et al.*, 1982), followed by extensive dialysis against 5 mM-Tris/HCl, pH 8.0, containing 0.5 mM-EDTA. Linear plasmid templates for transcription were prepared by *Bam*H1 or *Eco*RI digestion, phenol extraction, ethanol precipitation and resuspension in 5 mM-Tris/HCl (pH 8.0)/0.5 mM-EDTA. Transcription of both supercoiled and linear plasmid templates was carried out in the buffer described by Melton *et al.* (1984), with DNA at 50 ng/ml, RNasin at 4000 units/ml and SP6 RNA polymerase at 500 units/ml. The reaction initially contained 0.5 mM each of ATP, CTP and UTP, 0.005 mM-GTP and 0.25 mM-m^{7}GpppG. After 10 min incubation at 39 °C, the concentration of GTP was increased to 0.5 mM, and incubation continued for a further 50 min (Stueber *et al.*, 1984). After transcription, the products were analysed by agarose-gel electrophoresis in the presence of 90 mM-Tris/borate (pH 8.3)/0.25 mM-EDTA. Aliquots of the transcription reaction were either translated directly in a wheat germ system (Roberts & Paterson, 1973) or stored at −80 °C for subsequent translation. The volume of transcription reaction added was one-tenth that of the final volume of the wheat germ reaction, with the Mg^{2+} content adjusted accordingly. The final concentrations of components in the wheat germ reactions were 20 mM-Hepes/KOH, pH 7.6, 8 mM-Tris/acetate, pH 7.6, 4 mM-Tris/HCl, pH 7.5, 120 mM-potassium acetate, 1.25 mM-magnesium acetate, 0.6 mM-$MgCl_2$, 0.45 mM-spermidine, 1.05 mM-ATP, 0.1 mM-GTP, 0.05 mM-CTP, 0.05 mM-UTP, 0.025 mM-m^{7}GpppG, 8 mM-creatine phosphate, 100 μg of creatine kinase/ml, 3.0 mM-dithiothreitol, 0.025 mM each of 19 amino acids (not methionine), 10 μg of bovine serum albumin/ml, 50 units of SP6 RNA polymerase/ml, 400 units of RNasin/ml, 200 μCi of L-[^{35}S]methionine/ml (1200 Ci/mmol) and 10 mg of wheat germ protein/ml. At 26 °C protein synthesis was completed within 60 min, after which time aliquots were removed for determination of the incorporation of [^{35}S]methionine into protein, and for processing and chloroplast import experiments.

Chloroplast preparations

Intact chloroplasts were purified from leaves of 10-day-germinated *Pisum sativum* L. var. Feltham First, by Percoll gradient centrifugation (Robinson & Ellis, 1984*b*). A stromal extract was prepared by osmotic rupture of chloroplasts in 20 mM-Tris/HCl, pH 7.6, followed by centrifugation at 12000 *g* for 5 min to remove membranes. Proteolytic processing of proteins synthesized *in vitro*, and import into intact chloroplasts, were carried out as described by Robinson & Ellis (1984*a*,*b*).

Polyacrylamide-gel electrophoresis of chloroplast proteins

Polypeptides labelled with [^{35}S]methionine were analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) and autoradiography. To improve resolution of low-M_r polypeptides, gels were comprised of gradients of acrylamide and bisacrylamide [from 10% (w/v) acrylamide/0.267% (w/v) bisacrylamide to 30% (w/v) acrylamide/0.2% (w/v) bisacrylamide]. Gels were stained in 0.02% Coomassie Blue R-250 in acetic acid/methanol/water (7:40:53, by vol.) and destained in the same solution without Coomassie Blue.

RESULTS

Construction of plasmids to direct the synthesis of pS, S and TP

In order to begin our investigation of the mechanism of import of RuBP carboxylase small subunit (S) into chloroplasts, we chose to employ an SP6 transcription system (Krieg & Melton, 1984) with a cell-free protein-synthesizing extract from wheat germ (Roberts & Paterson, 1973). Apart from synthesizing the small subunit precursor, pS, we also wished to synthesize TP and S polypeptides independently, in order to determine in which ways each contributes to the functioning of the precursor during import and processing by chloroplasts.

The DNA sequence employed to direct the synthesis of S is derived from a cDNA clone, since the genomic sequences contain introns (Cashmore, 1983). The cDNA clone employed, pGR407, was constructed from two partial cDNA clones (pSSU1 and pSSU69) which have been described previously (Bedbrook *et al.*, 1980). The sequence of the cDNA of pGR407 is given in Fig. 1(*a*), since the original sequence presented (Bedbrook *et al.*, 1980) contains mistakes which alter 12 amino acid assignments. The amino acid sequence of the S polypeptide encoded by pGR407 is identical with that encoded by the cDNA clone pSS15 and the genomic clones pPS-2.4 (Coruzzi *et al.*, 1984) SS3.6 and SS8.0 (Timko *et al.*, 1985) which were isolated from a different cultivar of *Pisum sativum*. Within the cDNA sequence of pGR407, a single *Sph*1 restriction endonuclease site precisely separates the information for TP from that for S. This site was used to clone the DNA fragment encoding S from pGR407 into the SP6 expression

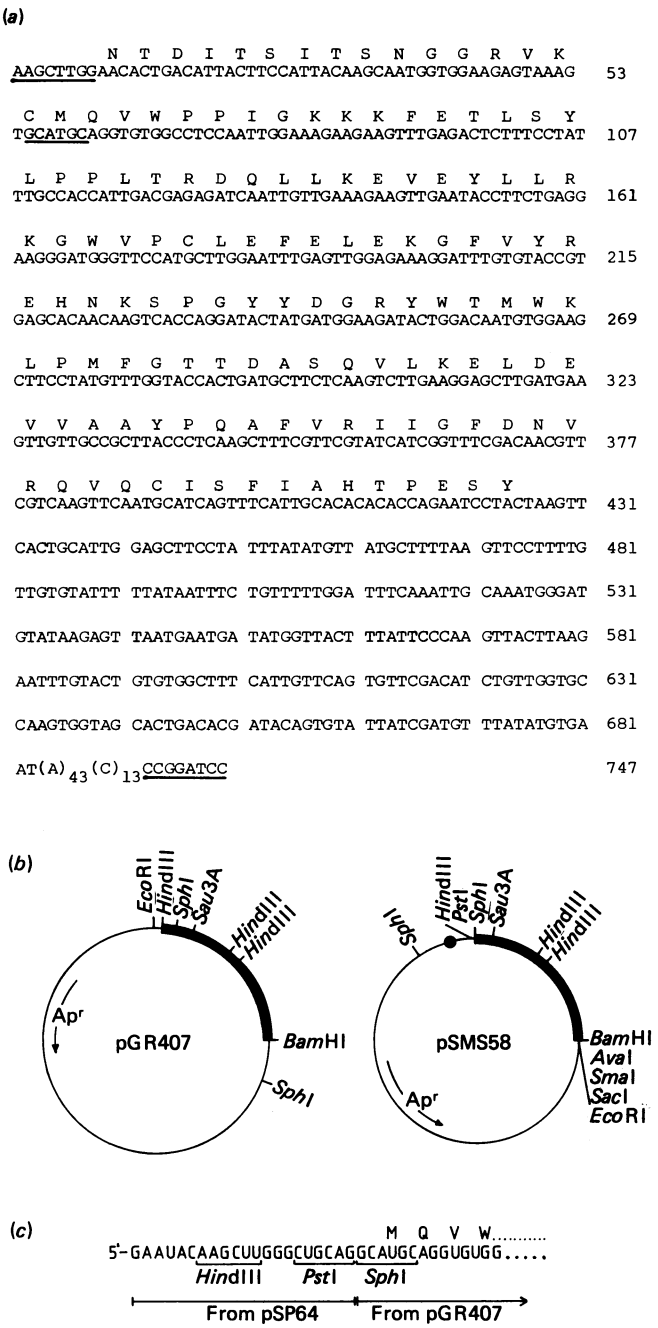

Fig. 1. Nucleotide sequences and plasmids encoding S

(*a*) DNA sequence of the non-coding (mRNA-sense) strand of the cDNA insert of pGR407 with the encoded amino acid sequence shown in single-letter notation above. The terminal nucleotides underlined correspond to *Hin*dIII and *Bam*H1 linker molecules used in the cloning procedures, and the *Sph*1 site which separates the information for TP and S is also underlined. The 3′ end of the sequence also contains 43 adenine residues derived from the poly(A) tail and 13 cytosine residues added during the initial cDNA cloning. (*b*) Structures of pGR407 and pSMS58. The cDNA is shown as a filled bar, and the SP6 promoter as a filled circle. (*c*) Sequence of the 5′ end of the SP6 transcript from pSMS58. The relationship between this sequence and the restriction enzyme sites shown in (*b*) is indicated below, together with an indication of the plasmid origin of this sequence. The first four amino acid residues shown correspond to the *N*-terminus of S (Bedbrook *et al.*, 1980).

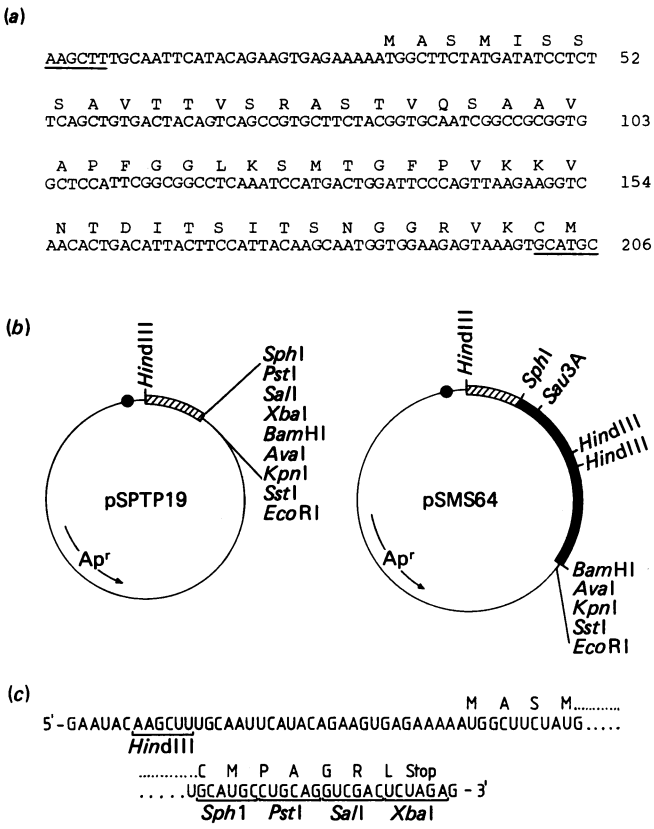

Fig. 2. Nucleotide sequences and plasmids encoding pS and TP

(*a*) DNA sequence of the non-coding (mRNA-sense) strand of the DNA fragment encoding TP, with the encoded amino acid sequence shown in single-letter notation above. The nucleotides underlined correspond to the *Hin*dIII and *Sph*1 restriction enzyme sites which define this fragment. The methionine residue encoded within the *Sph*1 site represents the *N*-terminal amino acid residue of S. (*b*) Structures of pSPTP19 and pSMS64. The sequence encoding TP is shown as a hatched bar and that encoding S as a filled bar. The SP6 promoter is shown as a filled circle. (*c*) Sequence of the 5′ end of the SP6 transcripts from pSPTP19 and pSMS64 and (below) the sequence from pSPTP19 which encodes the *C*-terminal amino acid residues of TP. The relationships between these sequences and the restriction enzyme sites shown in (*b*) are indicated below each sequence.

plasmid pSP64 (Melton *et al.*, 1984). The resulting plasmid, pSMS58, is shown in Fig. 1(*b*) (see the Materials and methods section for further details of its construction). The SP6 transcript expected from pSMS58 is presented in Fig. 1(*c*) and shows that the first AUG codon from the 5′ end of the RNA is that which encodes the *N*-terminal methionine residue of S. The nucleotide three positions upstream of the AUG codon is a purine, which would be expected to allow efficient translation by eukaryotic ribosomes (Kozak, 1984).

Since our cDNA clone does not encode a complete TP sequence, we obtained the necessary DNA fragment from a genomic clone. This *Hin*dIII–*Sph*1 fragment contains 31 nucleotides of 5′ non-translated sequence followed by the coding information for TP (Fig. 2*a*). The deduced amino acid sequence of this TP is consistent with others described for this protein from pea: the sequence of the 57 amino acids differs in only one residue

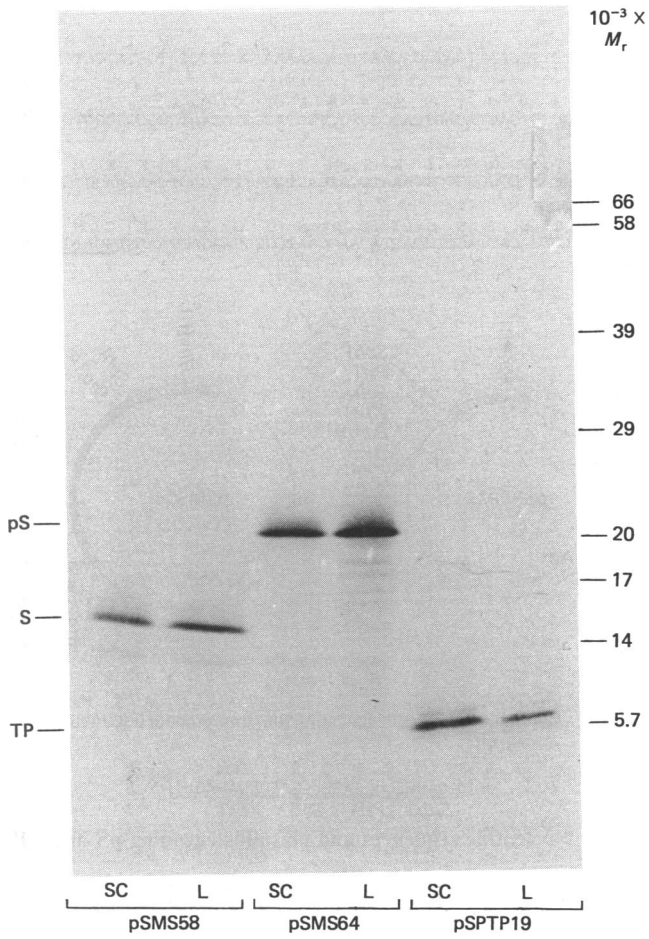

Fig. 3. Synthesis of S, pS and TP from SP6 transcripts

Plasmid DNAs pSMS58, pSMS64 and pSPTP19 were transcribed using SP6 RNA polymerase, both in supercoiled form (SC) and after linearization (L) with *Bam*HI. After transcription, 2.5 μl of each reaction mixture was added to a 25 μl wheat germ translation reaction containing L-[^{35}S]methionine. Polypeptides synthesized were visualized by SDS/polyacrylamide-gel electrophoresis and autoradiography. M_r markers, shown on the right, are: bovine serum albumin (66000), catalase (58000), aldolase (39000), carbonic anhydrase (29000), trypsin inhibitor (20000), myoglobin (17000), lysozyme (14000) and bovine insulin (5700).

from that of SS8.0 (Timko *et al.*, 1985) and in two residues from those of pPS-2.4 (Coruzzi *et al.*, 1984) and SS3.6 (Cashmore, 1983). This *Hin*dIII–*Sph*1 fragment was cloned together with the *Sph*1–*Bam*H1 fragment encoding S (Fig. 1), into pSP64 to produce pSMS64, which encodes pS (Fig. 2*b*, and see the Materials and methods section). The same *Hin*dIII–*Sph*1 fragment was subsequently also cloned into a derivative of pSP64 to produce pSPTP19 which encodes only TP (Fig. 2*b*, and see the Materials and methods section for details).

The SP6 transcripts expected from pSPTP19 and pSMS64 will have the same 5′ end, the first AUG codon will be that of the precursor polypeptide, and the nucleotide three positions upstream of the AUG codon is a purine (Fig. 2*c*). The transcript derived from pSPTP19 encodes the transit peptide of 57 amino acids together with six residues at the *C*-terminal end derived from the vector cloning site (Fig. 2*c*).

Synthesis of pS, S and TP

Transcription reactions employ either linear plasmid DNA templates (Krieg & Melton, 1984; Mead *et al.*, 1985) or supercoiled plasmid DNAs. The transcription reactions contain m^{7}GpppG to cap transcripts at the 5′ end, and so allow efficient translation subsequently (Stueber *et al.*, 1984). Aliquots of the transcription reaction are added directly to a wheat germ cell-free system without any prior DNAase treatment or phenol extraction. The Mg^{2+} content of the wheat germ system is modified to allow for the contribution from the added transcription reaction products. Fig. 3 shows the result of transcribing similar amounts of both supercoiled and linear template for each of the three constructs described above, followed immediately by translation and SDS/polyacrylamide-gel electrophoresis. The products of translation are quantitatively and qualitatively similar, regardless of the form of the transcription template. Similarly, phenol extraction and ethanol precipitation of the transcription reaction products does not significantly affect the quantity or quality of translation products finally obtained (results not shown).

Fig. 3 shows that our constructs in SP6 plasmids direct the synthesis of polypeptides of the anticipated M_r values. The precursor polypeptide, pS (M_r 20208), comigrates precisely with the authentic molecule produced in response to pea leaf RNA (result not shown), and the mature polypeptide, S (M_r 14496), comigrates with that isolated from pea leaves (see below). The transit peptide has not previously been observed, but the molecule which we have synthesized migrates a little slower than does bovine insulin (Fig. 3) in agreement with its calculated M_r of 6355 (insulin has M_r 5709).

Polypeptide processing by stromal proteinase(s)

The polypeptides synthesized *in vitro* were incubated post-translationally with a stromal extract prepared from chloroplasts purified by Percoll-gradient centrifugation (Fig. 4*a*). The pS molecule is processed to one which comigrates with S, while S synthesized from pSMS58 is resistant to proteolysis. The S molecule produced by processing pS, and that synthesized in response to pSMS58, each comigrates precisely with the S molecule present in the stromal extract, and visible on the stained polyacrylamide gel. The pS and S polypeptides are immunoprecipitable with a serum raised against RuBP carboxylase (result not shown). These observations support our conclusion that our plasmid constructs direct, with fidelity, the synthesis of these two polypeptides. The TP molecule is greatly reduced in amount upon incubation with stromal extract, but the products of this degradation process are not observed (Fig. 4*a*).

It has been reported that during the processing of pS, TP is cleaved from S in two steps (Robinson & Ellis, 1984*b*), by a proteinase which is inhibited by the metal-ion chelators, EDTA and 1,10-phenanthroline (Robinson & Ellis, 1984*a*; see also Fig. 4*b*). The degradation of TP by stromal extract is also inhibited by EDTA and 1,10-phenanthroline (Figs. 4*b* and 4*c*). We believe therefore that the observed degradation of TP by stromal extract may reflect a physiologically significant process. In this case, we might expect to see TP undergo a single endoproteolytic cleavage at short time points

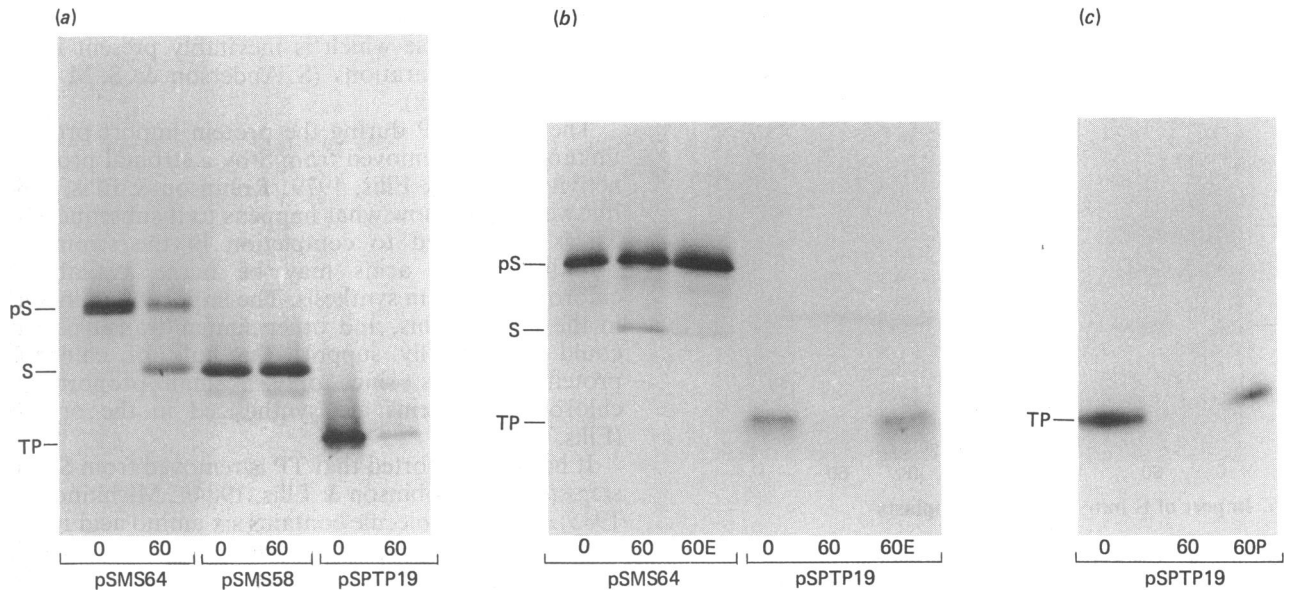

Fig. 4. Proteolytic processing of pS and TP by stromal extract

Polypeptides synthesized in reponse to DNAs pSMS64, pSMS58 and pSPTP19 were incubated post-translationally with stromal extract and analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. (*a*) Samples were analysed before the addition of stroma (0) and after 60 min incubation (60). (*b*) Polypeptides synthesized from pSMS64 and pSPTP19 were incubated for 60 min in the absence (60) and presence (60E) of 5 mM-EDTA. (*c*) The transit peptide synthesized from pSPTP19 was incubated for 60 min in the absence (60) and presence (60P) of 5 mM-1,10-phenanthroline.

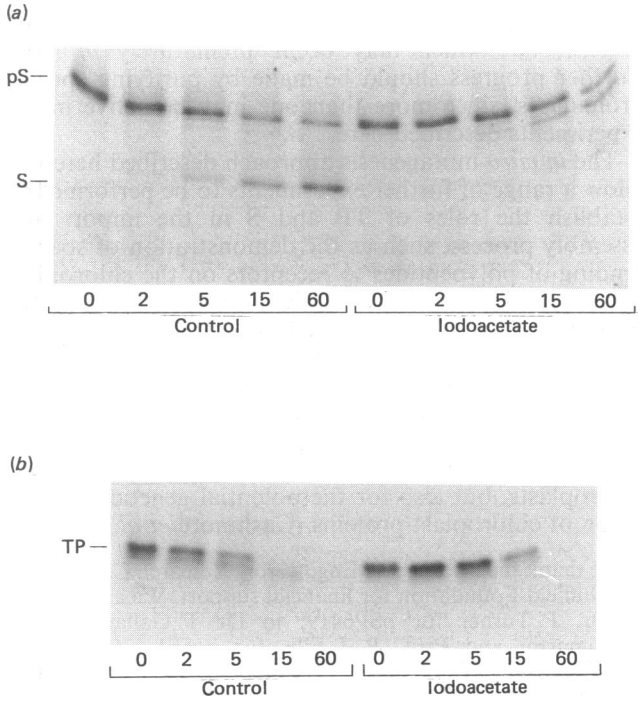

Fig. 5. Inhibition of proteolytic processing by iodoacetate

pS and TP were synthesized from pSMS64 (*a*) and pSPTP19 (*b*) respectively, and incubated on ice with or without 10 mM-iodoacetate for 30 min. An equal volume of stromal extract was subsequently added and incubated at 27 °C. Samples were removed at intervals of 0, 2, 5, 15 and 60 min as indicated, boiled with 2% (w/v) SDS for 2 min, and analysed by gel electrophoresis and autoradiography.

during incubation with stromal extract (Robinson & Ellis, 1984*b*). We therefore incubated TP, and for comparison, pS, for different time intervals with stromal extract. The processing of pS to S is incomplete after 60 min (Fig. 5*a*), while no TP remains after this time (Fig. 5*b*). At short time intervals (2–15 min) during such processing, we see an increase in the mobility of TP which corresponds to the removal of a small number of amino acid residues (Fig. 5*b*). A possible explanation for this observation is that the stromal proteinase activity rapidly removes the six *C*-terminal amino acids by cleaving between Cys and Met residues. The cleavage between these residues in pS (separating TP from S) is inhibited by iodoacetate, whereas cleavage within TP is not sensitive to iodoacetate. Thus, in the presence of iodoacetate, pS is processed to a molecule of M_r approx. 18000 (Robinson & Ellis, 1984*b*, and Fig. 5*a*). Similarly, we find that iodoacetate prevents the rapid increase in mobility of TP but does not prevent its subsequent degradation (Fig. 5*b*). These observations lead us to believe that the rapid increase in mobility of TP in the absence of iodoacetate is due to the removal of the six *C*-terminal amino acids by cleavage between the Cys and Met residues. No subsequent products of TP degradation are seen (Fig. 5*b*). Further analysis of such products requires methods for the detection of small peptides. Our gel electrophoresis system is suitable for the detection of the B-chain of insulin (30 amino acid residues, M_r 3396) but the A-chain (21 amino acid residues, M_r 2330) is lost during fixation in acetic acid and methanol. Thus, the products of a single endoproteolytic cleavage within TP may go undetected in our system. Alternatively, the activity of other proteinases in the stromal extract may immediately remove such products.

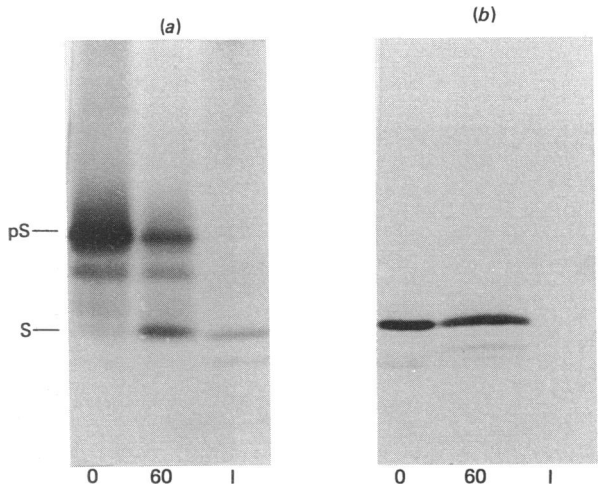

Fig. 6. Import of S into isolated chloroplasts

pS and S were synthesized from pSMS64 (*a*) and pSMS58 (*b*) respectively, and incubated with chloroplasts purified by Percoll gradient centrifugation, for 60 min with illumination. Polypeptides present at the beginning of the incubation (0), those present at the end (60) and those imported into the chloroplasts (I) were analysed by gel electrophoresis and autoradiography.

Import of S into isolated chloroplasts

The construction of plasmids directing the synthesis of pS and S allows us to test the hypothesis that the TP is necessary for import into the chloroplast. After incubation of pS with purified intact chlorplasts in the light, S is found inside the chloroplasts (i.e. it is resistant to added trypsin) while the remaining pS is not imported (Fig. 6*a*). On the contrary, when S is incubated with isolated chloroplasts, none can be detected which is trypsin-resistant (Fig. 6*b*). We are unable to determine if TP alone is capable of being imported, since we know of no treatment which will inhibit its degradation, and still leave the import process unimpaired.

DISCUSSION

We have constructed plasmid molecules which encode pS, S and TP, and have synthesized these molecules in cell-free systems employing SP6 RNA polymerase and wheat germ extract. The constructs have enabled us to demonstrate that removal of TP from pS abolishes the ability of isolated chloroplasts to import S. The same conclusion has been reached by Mishkind *et al.* (1985) using a partially purified proteinase from *Chlamydomonas* to prepare the algal S molecule from pS. Isolated pea chloroplasts will import pS but not S. The present study demonstrates that the same is true when polypeptides and chloroplasts from the same organism are examined. A plausible explanation for these observations is that TP is necessary for the import of S into chloroplasts. This explanation is consistent with the observation that when TP is fused to a bacterial protein, the latter can be imported into chloroplasts (Van den Broeck *et al.*, 1985). However, we feel that it will be important in future to demonstrate that mature S cannot be imported into chloroplasts *in vivo*, since *in vitro* systems have the potential to introduce artefacts. For example, the inability of isolated chloroplasts to import mature S might be a consequence of the ability of S to adhere to RuBP carboxylase which is inevitably present in such chloroplast preparations (S. Anderson & S. M. Smith, unpublished work).

The fate of TP during the protein import process is unknown. It is removed from S by a stromal proteinase activity (Smith & Ellis, 1979; Robinson & Ellis, 1984*a*) but we do not know what happens to it subsequently. If TP is hydrolysed to completion in the stroma, the resulting amino acids may be made available for chloroplast protein synthesis. The import of amino acids in the form of this, and other similar transit peptides, could theoretically support the bulk of chloroplast protein synthesis, since only a small proportion of chloroplast proteins are synthesized in the organelle (Ellis, 1981).

It has been reported that TP is removed from S in two steps *in vitro* (Robinson & Ellis, 1984*b*; Mishkind *et al.*, 1985). Our TP molecule contains six amino acid residues at the *C*-terminal side of the cysteine residue at which the second endoproteolytic cleavage is thought to take place. However, during incubation with stroma we can detect an increase in mobility of TP which we believe to correspond to a proteolytic cleavage at this point, before any further proteolysis of TP occurs (Fig. 5*b*). While we would not suggest that our data provide evidence for a one-step removal of TP from pS, it does indicate that we should interpret with caution results obtained from such cell-free systems. We believe that with the limited data available, it may be premature to conclude that two-step processing occurs *in vivo*. For example, the endoproteolytic cleavage within TP could simply constitute a step in the proteolysis of TP after it has been removed from pS *in vivo*, but which may occur prematurely *in vitro*. Further progress should be made by purifying specific proteinases, since more than one may be active in the experiments described here.

The *in vitro* mutagenesis approach described here will allow a range of further experiments to be performed to establish the roles of TP and S in the import and assembly process, such as the demonstration of specific binding of polypeptides to receptors on the chloroplast envelope and the characterization of products of the proteolysis of TP. The construction of pSPTP19 provides a simple means of subsequently making fusions between TP and any other polypeptide sequence, to investigate the ability of TP to direct foreign proteins into the chloroplast. This information will be important not only for understanding how proteins are imported into chloroplasts, but also for the potential genetic manipulation of chloroplast proteins (Cashmore *et al.*, 1985).

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